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Award Number: DAMD17-00-1-0640

TITLE: Targeting Delivery of Chemotherapeutic Agents to Mammary Tumors

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REPORT DATE: April 2002

TYPE OF REPORT: Final Addendum

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2002	Final Addendum (1 Sep 01 - 3 Mar 02)

4. TITLE AND SUBTITLE
Targeting Delivery of Chemotherapeutic Agents to Mammary Tumors

5. FUNDING NUMBERS
DAMD17-00-1-0640

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains 6 color photos (Figures 1-6)

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Breast cancer is the second leading cause of cancer incidence and the leading cause of cancer mortality in women. Current chemotherapeutic treatments often have adverse effects primarily caused by the inefficient delivery and/or poor specificity of the compounds to breast tissues. Overall, we were interested in determining whether current therapeutic agents be redesigned to carry "tissue specific markers" to enhance their delivery and uptake in targeted tumor cells. Recently, researchers identified germline mutations in the tumor suppressor gene *BRCA1* that predisposes women to early onset breast cancer. To recapitulate this condition, our laboratory generated a Brca1 mouse model that selectively develops mammary tumors between 6-9 months of age. Using this Brca1 breast cancer model and cell lines derived from mammary tumors, phage display was used to isolate and identify peptide motifs that selectively bind to cultured Brca1 mammary tumor cell lines and were conjugated to a tracer. *In vitro* and *in vivo* efficiency and specificity of our candidate peptides toward mammary tumor cell lines and tissues was tested. The peptides were conjugated with TAT, which increased efficacy while decreasing specificity. Lastly, we identified the cellular localization of our candidate peptides.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast cancer, Brea 1, chemotherapy	19
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

Breast cancer is the most common cancer and the second leading cause of cancer mortality in women. Mutations in BRCA1 have been identified in 50 and 80 percent of familial cases of breast or breast/ovarian cancer, respectively. To gain further insite into this devastating disease, our laboratory has recently generated a mouse model for BRCA1 familial cancer with a unique disruption of BRCA1 in mammary epithelial cells (1). Tumorigenesis occurs at a low frequency after long latency, suggesting that multiple factors may be involved in BRCA1 related tumorigenesis. In addition to studying pathways dysregulated in hereditary breast cancer, our laboratory is also utilizing these mice as pre-clinical models to carry out novel chemoprevention studies.

The currently available chemotherapeutic treatments often have adverse effects primarily caused by the inefficient delivery and/or poor specificity of the compounds to breast tissues. Untoward effects such as myelosuppression and dose dependent cardiotoxicity are often associated with common chemotheraputic regimens. Many methods are currently under investigation to circumvent the side effects of systemic application of therapeutic agents. Recently, novel techniques such as protein, gene, and lyposomal therapies have been investigated as alternative delivery approaches for therapeutic agents. However, each method lacks efficiency and specificity for targeted tissues. Overall, we were interested in determining whether current therapeutic agents be redesigned to carry "tissue specific markers" to enhance their delivery and uptake in targeted tumor cells [Figure 1A]. In order to accomplish this goal, efficient delivery and increased specificity of chemotherapeutic agents to targeted cells must be achieved.

Recent data suggests tissue specific peptide motifs can be identified using phage display and biopanning (2). To identify tissue specific markers we used phage display, a selection technique where random peptides (7-mers) are incorporated into the genome of

bacteriophage and the random peptides expressed as part of the bacteriophage virion coat. In its simplest form, biopanning is carried out by incubating a library of phage-displayed peptides with your target of interest, washing away the unbound phage, eluting the specifically-bound phage, and amplification. Enrichment of the cell-associated phage is carried out with additional rounds of binding/amplification cycles which selects or favors sequences that selectively bind to the target [Figure 1B]. The resulting phage are sequenced and tested for binding.

Using this Brca1 breast cancer model and cell lines derived from mammary tumors, phage display was used to isolate and identify peptide motifs that selectively bind to cultured Brca1 mammary tumor cell lines. Next, the identified candidate peptides were conjugated to a tracer and tested for *in vitro* and *in vivo* efficiency and specificity of delivery toward mammary tumor cell lines and mammary tissues. We conjugated the candidate peptides to TAT binding sequence and tested for in vitro specificity and efficacy toward mammary cell lines and controls. Then, we identified the cellular localization of our candidate peptides in cultured cells.

Body

1. Candidate peptide selection.

To initiate this study, biopanning was performed to select peptides that bind Brca1 mammary tumor cell lines and fibroblasts, as controls. After the first round of biopanning, 3.4 x 10^6 colony forming units (CFUs) were recovered from the panned Brca1 cells and 1.65 x10^6 CFUs from fibroblasts that serve as biopanning controls [Figure 1C]. Two more rounds of phage amplification and biopanning selection resulted in the recovery of 13 x10^6 and 7.8 x 10^6 CFUs for the Brca1 cells and fibroblasts, respectively [Figure 1C]. Therefore, increased numbers of recovered CFUs with

successive rounds of biopanning and amplification suggests selective amplification of phage that bind to cells of interest.

Next, 100 clones isolated from the Brca1 display library were amplified and sequenced to initiate the screen and to determine whether the clones showed overlapping amino acid motifs. After data analysis, there were at least 6 recognizable amino acid motifs [Figure 1D]. None of the amino acid motifs or peptides identified in our Brca1 phage library was found in our control fibroblast display library, which was performed simultaneously with our Brca1 screening, or random clones isolated from our initial titer. These results suggest that the peptides may be selective for Brca1 tumor cells.

2. In vitro specificity and efficiency of candidate peptide delivery.

Next, we wanted to determine which of the peptides identified in our Brca1 phage library was specific for Brca1 tumor cells. In this set of experiments, the question was asked whether the identified candidate phage demonstrate uptake and specificity *in vitro*. Therefore, each of the amplified and isolated phage clones from the Brca1 display library was incubated with Brca1 tumor cells and fibroblasts to determine which amino acid motif and peptides were selective for Brca1 tumor cells [Figure 2A]. As shown in Figures 2B and C, phage clones A and B from our Brca1 display library also bound to our control fibroblasts which suggests that these phage clones can bind to a variety of tissues. However, many of the Brca1 display library clones (clones C-L) were more selective for Brca1 tumor cells [Figures 2B,C]. These results suggested that candidate peptides carrying the S/GLP amino acid motif would be the best test candidates for further characterization.

Next, we were interested in determining the most effective marker or tracer for our peptide delivery system. Here, we wanted demonstrate that the peptides, as opposed

to the original phage, could bind to cells. This could only be accomplished by identifying a tag that could be added to the peptides so they may be traced in our *in vitro* studies. Our selection of FITC as a tracer or marker, however never demonstrated uptake of FITC tagged peptides *in vitro*. This result could suggest that 1) the isolated phage clones do not bind to the cells. 2) the peptides derived from the phage library require the 3-dimentional structure or additional sequences from the phage coat protein for binding [Figure 3A]. 3) a linker is required to allow sufficient distance between the tracer and the peptide sequence for cell binding. 4) the hydrophobic nature of FITC prevents peptide binding to live cells.

In order to resolve some of the issues and demonstrate our Brca1 phage display library indeed identified peptides that were selective for our panned Brca1 tumor cells, we modified our tracer. Instead of using FITC as a tracer, we generated peptides that incorporated the HA tag into our peptide sequence. Several characterized tags with available antibodies could have been investigated, however the HA tag contained fewer negative charged residues which could potentially be repelled by the living cells [Figure 3B]. In addition, the length of linkers was also tested [Figure 3C].

Next, we were interested in determining whether our new tracer strategy indeed would accomplish our goals. Therefore, we incubated the HA-S/GLP-peptides with our cell lines. As shown in Figure 3D, our candidate peptides (HA-S/GLP-peptides) carrying the HA tag did not bind to fibroblasts. However, when the peptides were incubated with Brca1 tumor cells, we found uptake of the peptides which demonstrates that our tag system would be an excellent means to identify cells that are bound by our Brca1 peptides [Figure 3E and 3G].

3. *In vivo* specificity and efficiency of candidate peptide delivery.

Next, we ask whether our candidate peptides have specificity for targeting cells *in vivo*. Cell lines generated from our Brca1 mammary tumors were injected into nude mice. After tumors developed we injected our candidate peptides and/or controls into these mice and performed immunohistochemical analysis on a variety of tissues and tumors to determine the delivery of our peptides. In addition, mice with palpable mammary tumors were injected with our tagged targeted peptides. As shown in Figure 4, immunohistochemical analysis of normal appearing mammary [Figure 4B] and mammary tumors [Figure 4C] of mice injected with our tagged peptides demonstrated binding in regions of mammary epithelial cells. However, upon further analysis of other tissues from these mice suggests that we were detecting our tagged peptides that are retained in the blood stream, primarily bound to red blood cells [Figure 4D]. This data suggests that further investigations are required to determine methods of preventing binding of our peptides to serum cells/proteins while allowing the peptides access to mammary tumor cells. The best method of accomplishing these goals would probably be with combined use of our peptide system in addition to polymers or sterically stabilized liposomes.

4. *In vivo* specificity and sensitivity of candidate peptides carrying therapeutic agents.

In this series of experiments, we wanted to determine whether the candidate peptides conjugated to Doxorubicin (Adriamycin) has the ability to inhibit tumor growth. First we were interested in determining whether Doxorubicin had the ability to kill our Brca1 breast tumor cells. As shown in Figure 4E, our Doxorubicin dose curve demonstrates that over 50% of our cultured Brca1 tumor cells do not survive at 0.1 μg/ml. This data suggests that when we have overcome the non-specific binding of our

peptide to serum red blood cells, that Doxorubicin may be an excellent candidate for our future *in vivo* studies.

5. *In vitro* specificity of candidate peptides conjugated to TAT peptide sequence.

Next, we asked whether the efficiency of our "tagged" candidate peptides could be increased by conjugating our peptides to the TAT peptide sequence (3). In theory, the aim was to increase the amount of candidate peptide that could be taken up by our target cells and tissues. Therefore, we generated polypeptides in three combinations [Figure 5]. One of the peptides carried the TAT sequence directly conjugated to our candidate peptide [Figure 5A]. Another peptide carried a linker placed between the candidate peptide and TAT [Figure 5B]. The third peptide carried an additional linker placed between the candidate peptides and TAT [Figure 5C].

Next, we asked whether our candidate peptides conjugated to TAT demonstrated sensitivity toward our targeted cultured cells. Therefore, we incubated our newly generated TAT conjugated peptides (from Figure 5) with our Brca1 cultured cells. As shown in Figure 5 D and E, we observed binding of our peptide to our cells of interest. However, as control, we also incubated the peptides with fibroblasts and found binding of the TAT peptides to the cells, even with short incubations [Figure 5F and G]. These data suggest that the specificity conferred by our original (S/GLP) candidate peptide was lost when the peptide was conjugated to the TAT sequence. However, comparing the short incubations of the TAT conjugated peptides to the Brca1 tumor cells and fibroblasts, it appears that the TAT peptide acted in concert with our candidate peptide sequence to increase the uptake of our tracer. Although some specificity was lost, the uptake of the tracer in Brca1 tumor cell lines was increased (compared to fibroblasts) using the TAT peptide sequence [Figure 5 E and G].

6. Cellular localization of our candidate peptide sequences to the plasma membrane and nucleus of Brca1 cultured cells.

One observation that was made from the data generated in Figure 5 was that the TAT conjugated peptides bound to the plasma membrane of our Brca1 cell lines [Figure 5E]. However, in fibroblasts, the same peptides appeared to be in the cytoplasm, and specifically localized in cytoplasmic organelles [Figure 5G]. This may suggest that the TAT sequence increases internalization of the peptides. Therefore, we asked the question of where do our candidate peptides reside within the cells. Therefore, we incubated our HA-S/GLP-peptides that demonstrated specificity toward Brca1 cultured cells. Using, immunoflourescence were able to demonstrate that some peptides bound to the plasma membrane with some increased areas of local binding as demonstrated by increased intensity of signal on the plasma membrane[Figure 6 A-F]. While other peptides appear to traverse the plasma membrane and go directly into the nucleus [Figure 6 G-I]. These data may lead to other possible uses of peptides for drug delivery. If the mechanism of action for a specific drug requires localization to the plasma membrane, cytoplasmic organelles or nucleus, perhaps a specific peptide conjugated to that drug would increase the efficacy just by locally increasing the concentration of the drug to that organelle.

Key research accomplishments

Overall, this study provided an investigation of using phage display as a means of identifying peptides that could be used as a means of selectively targeting drugs to tumors. In this study, we identified specific motifs that bound to Brca1 tumor cells [Figure 1D]. Second, we demonstrated the specificity of these motifs for Brca1 tumor cells [Figure 2B and C]. We identified the appropriate "tag" system that could be used to

trace the *in vitro* [Figure 3] and *in vivo* [Figure 4] distribution of our candidate peptides. We demonstrated that conjugating our peptide TAT peptide increases the efficiency of delivery with loss of specificity [Figure 5]. Lastly, we identified the localization of our candidate peptides to specific cellular regions [Figure 6] and speculate that perhaps chemotherapeutic agents can be modified using peptides to increase the cellular localization of the therapy.

Reportable outcomes

We have published the chemotherapeutic response of our Brca1 tumor cell lines to various agents (Brodie et al. Oncogene) . This data suggests that the Brca1 tumor cell lines are Tamoxifen resistant but highly sensitive to Doxorubicin or γ-irradiation, demonstrating that Doxorubicin would be the best chemotherapeutic candidate for future targeting studies.

Brodie S.G., Xu, X., Qiao W., Li. W., Cao L., Deng, C.X., (2001). Multiple genetic changes are associated with mammary tumorigenesis in Brca1 conditional knockout mice. **Oncogene 20 (51)**;7514-7523.

Conclusions

Our goal was to develop a highly specific and efficient method to deliver drugs to tumors. In this series of experiments we were interested in determining whether phage display could be used to identify peptides that could act as a beacon to target chemotheraputic agents to tumor cells. This could effectively increase the local dose of the drug in tumors. The results of this study suggests that this technique is viable and allowed the identification of candidates that could be tested both *in vivo* and *in vitro*. We also identified the linker length and identified the "tag" that could be conjugated to the

peptides for tracing our carrier molecules *in vitro* and *in vivo*. We also found that further studies are required to prevent the nonspecific binding of our peptides to serum components. We also demonstrated that the TAT sequence can increase the uptake of our candidate peptide to our cells of interest, however, specificity is lost using this sequence. We also identified the cellular localization of our candidate peptide sequence and speculate that perhaps these techniques could also be used to target specific therapeutic agents to the cellular organelle of interest. This new data may suggest that agents requiring nuclear or cytoplasmic localization may be targeted to specific organelles using this technique. Upon accomplishment of the proposed future experiments we will be able to determine whether targeted delivery of therapeutic agents is viable as a breast cancer treatment. Moreover, if targeted therapeutic treatment increases the efficacy and specificity of chemotheraputic agents then perhaps this concept may lead to treatments for other forms of cancer.

References

1) Xu, X. et al. (1999) Conditional mutation of Brca1 in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. Nat Genet 22, 37-43.

2) Arap W. et al (1998) Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science 279, 377-380.

3) Schwarz, S.R. et al. (1999) In vivo protein transduction: Delivery of a biologically active protein into the mouse. Science 285, 1569-1572.

Appendices

Figure 1. Phage display and biopanning used for candidate peptide selection.
(**A**) Diagram of the overall goal of the proposed research. (**B**) Diagram showing the technique of biopanning. (**C**) Graph demonstrating the numbers of phage recovered (CFU's) with sucessive round of biopanning. (**D**) Peptide motifs and sequences identifed from the Brca1 display library.

Figure 2. In vitro specificity and efficiency of Brca1 display phage.
(**A**) Diagram of the overall goal invitro specificity and efficiency of candidate phage. (**B**) Plate showing the means of identifying colony forming units (CFU's). (**C**) Graph demonstrating the numbers of phage recovered (CFU's) from specificity assay.

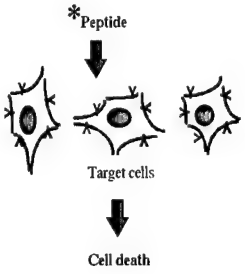
Figure 3. In vitro specificity and efficiency of candidate peptide and tag delivery.
(**A**) Protein sequence of phage coat protein surrounding the peptides. (**B**) Peptide sequences of various "tags" which could be utilized. (**C**) Dipection of our candidate constructs showing our peptide-linker-tag sequence. (**D-G**) Immunohistochemical staining for our candidate peptides incubated with fibroblasts (**D**), Brca1 cells (**E and G**). (**F**) Negative control

Figure 4. In vivo specificity and efficiency of tagged candidate peptides.
(**A-D**) Immunohistochemical staining for our candidate peptide after injection into Brca1 mammary tumor mice. Arrows point to positive staining in mammary tissue (B), mammary tumor (**C**) and Liver (**D**). (**A**) Negative control.

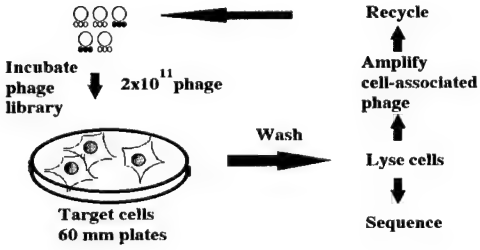
Figure 5. *In vitro* specificity of candidate peptides conjugated to TAT peptide sequence.
(**A-C**) Peptide sequences of various combinations of TAT conjugated peptides utilized in this series of experiments. (**D-G**) Immunohistochemical staining for our candidate peptides incubated for 10 minutes with Brcal cell lines (**D and E**) and fibroblasts at low (**F**) and high magnification (**G**).

Figure 6. Cellular localization of our candidate peptide sequences to the plasma membrane and nucleus of Brca1 cultured cells. (**A-I**) Immunoflourescence detection of our HA tagged candidate peptides bound to Brac1 cell lines. (**A, D, G**) DAPI staining. (**B, E, H**) Alexa Flour 488 conjugated secondary antibody (Green). (**C, F, I**) Merged sequence. Note the localization to the plasma membrane (**B, C, E, F**) and nuclear staining (**H and I**).

1A



1B



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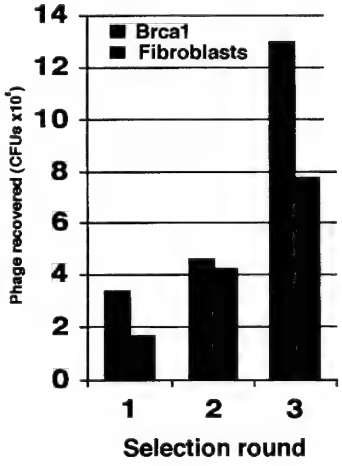


1D

MOTIF	Peptide sequence	PEPTIDE
SSAXL	ASHSSAPL	A
	SSALLSR	G
P/VPP	VPPQLFG	B
	VPPRLPV	K
S/GLP	VPSLPFP	C
	SLPIATR	E
	QNNSLPF	F
	GLPPPQR	H
	GLPTHQL	D
KQ	GFGSKQT	I
	AKRTKQY	J
SYX	SYVQYPH	L

Figure 1

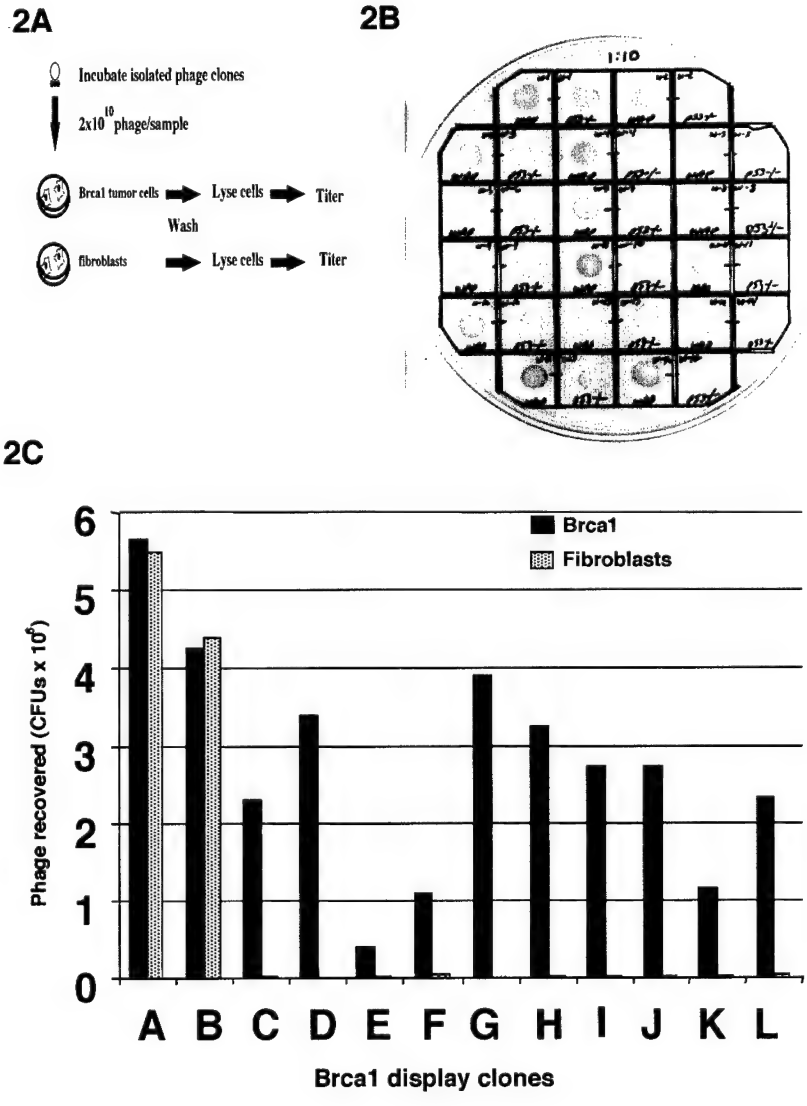

2A
Incubate isolated phage clones
2x10^10 phage/sample
Brca1 tumor cells
Lyse cells
Titer
Wash
fibroblasts
Lyse cells
Titer
2B
1:10
2C
Brca1
Fibroblasts
Phage recovered (CFUs x 10^6)
0
1
2
3
4
5
6
A B C D E F G H I J K L
Brca1 display clones

Figure 2

3A .....PFYSHS-XXXXXXX-GGSA.....

3B

Tag	Peptide sequence
Flag	MDYKDDDDK
c-Myc	MEQKLISEEDL
HA	YPYDVPDYASL

3C

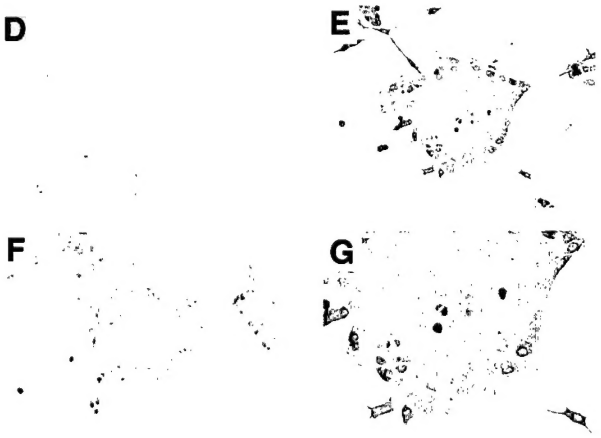
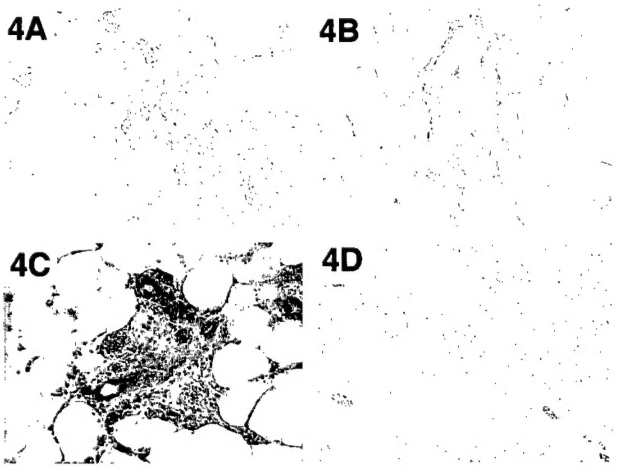


Figure 3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4B
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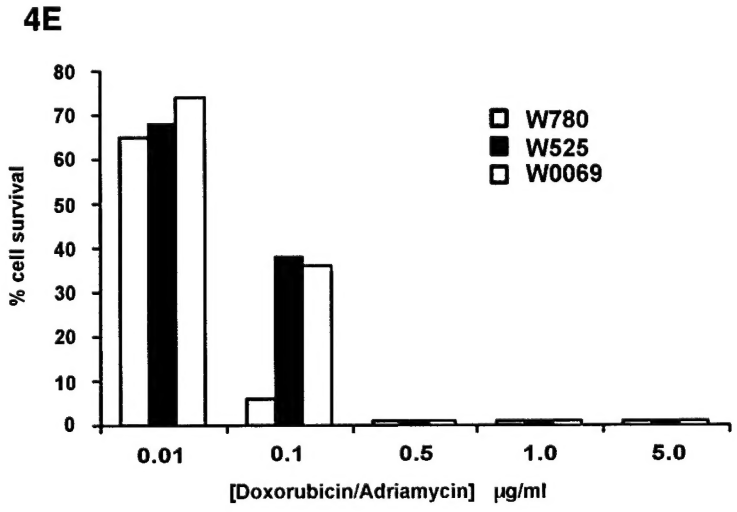
4E
% cell survival
80
70
60
50
40
30
20
10
0
0.01
0.1
0.5
1.0
5.0
[Doxorubicin/Adriamycin] µg/ml
W780
W525
W0069

Figure 4

5

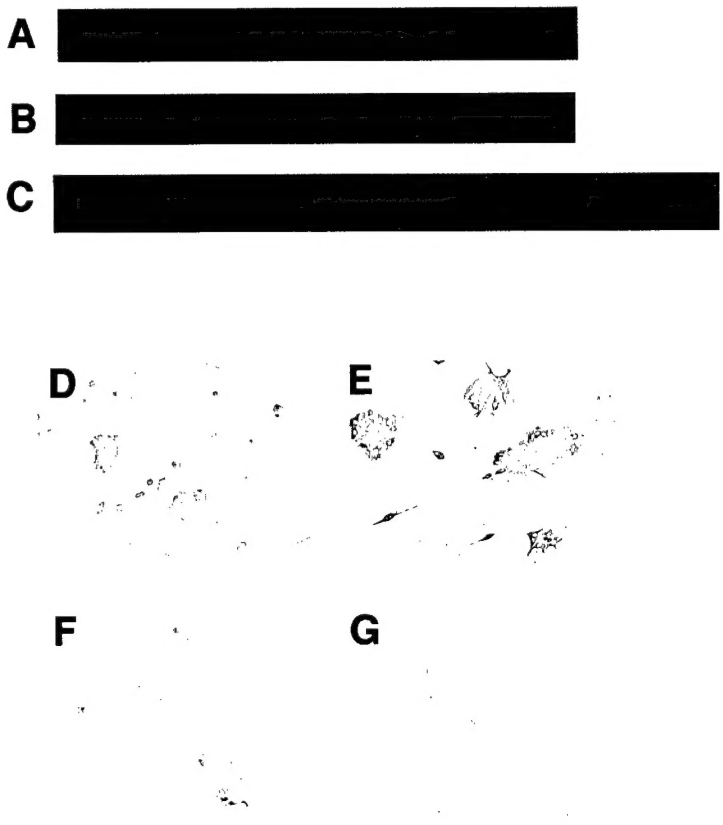

Figure 5

6

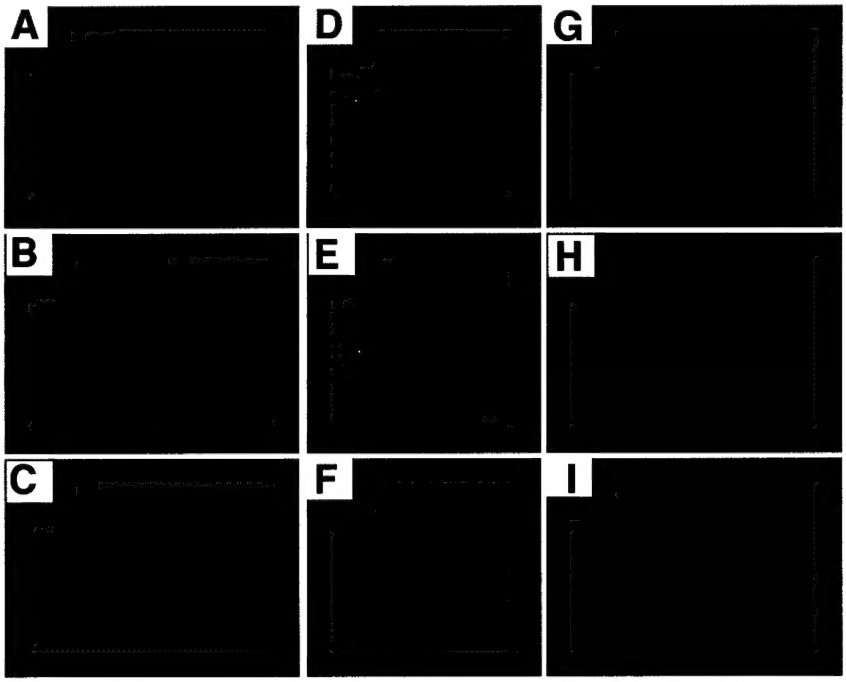

Figure 6